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Purification and Characterization of a Glycerol-Resistant CF_0-CF_1 and CF_1-ATPase from the Halotolerant Alga *Dunaliella bardawil*[1]

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ABSTRACT

The isolation of the chloroplast ATP synthase complex (CF_0-CF_1) and of CF_1 from *Dunaliella bardawil* is described. The subunit structure of the *D. bardawil* ATPase differs from that of the spinach in that the *D. bardawil* α subunit migrates ahead of the β subunit and ϵ-migrates ahead of subunit II of CF_0 when separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The CF_1 isolated from *D. bardawil* resembles the CF_1 isolated from *Chladmydomonas reinhardi* in that a reversible, Mg^{2+}-dependent ATPase is induced by selected organic solvents. Glycerol stimulates cyclic photophosphorylation catalyzed by *D. bardawil* thylakoid membranes but inhibits photophosphorylation catalyzed by spinach thylakoid membranes. Glycerol (20%) also stimulates the rate of ATP-P_i exchange catalyzed by *D. bardawil* CF_0-CF_1 proteoliposomes but inhibits the activity with the spinach enzyme. The ethanol-activated, Mg^{2+}-ATPase of the *D. bardawil* CF_1 is more resistant to glycerol inhibition than the octylglucoside-activated, Mg^{2+}-ATPase of spinach CF_1 or the ethanol-activated, Mg^{2+}-dependent ATPase of the *C. reinhardi* CF_1. Both cyclic photophosphorylation and ATP-P_i exchange catalyzed by *D. bardawil* CF_0-CF_1 are more sensitive to high concentrations of NaCl than is the spinach complex.

Dunaliella bardawil is a halotolerant, cell wall-less alga which is capable of surviving in growth medium containing NaCl ranging in concentration from 0.5 to 4 M. This is made possible by the synthesis of an internal osmoticum which appears to be mainly glycerol (5, 7, 10). Thus, the internal concentration of glycerol in the cell could range from 1 to 7.5 M (equivalent from 7.5 to 55 volume per cent) at the physiological conditions in which the alga survives. Such unusually high glycerol concentrations undoubtedly alter the properties of the solution inside the cell and, therefore, one might expect to find special adaptations of enzymic systems inside the alga that enable it to function under these unusual conditions. However, few comparisons between *D. bardawil* and other higher plant enzymes with respect to their sensitivity to glycerol have been reported.

In this work, we describe the isolation and characterization of the chloroplast CF_0-CF_1[3] ATP synthase and of the soluble CF_1 from *D. bardawil*. Although CF_1 has previously been isolated from a large number of higher plants (20) as well as from a green alga (27) and cyanobacteria (9), an active CF_0-CF_1 complex has only been isolated from higher plants (23). We demonstrate here that the CF_0-CF_1 ATPase from *D. bardawil* differs from its higher plant counterpart in its subunit structure, resistance to glycerol, and sensitivity to NaCl, indicating that the structure of the protein has been modified in order to enable it to function at high glycerol concentrations.

MATERIALS AND METHODS

Thylakoid Membranes Preparations. *Dunaliella bardawil* was grown outdoors in large (30 L) continuous cultures in a medium containing 1.5 M NaCl and nitrate as previously described (8). *D. bardawil* thylakoid membranes were prepared by a modification of the procedure of M. Shefer (personal communication) as follows. Log-phase cultures were collected by centrifugation at 2,500*g* for 5 min, resuspended, and homogenized in buffer containing 20 mM Na-Tricine (pH 8.0) and 15% (v/v) glycerol, and collected by centrifugation at 12,000*g* for 10 min at 5°C. The glycerol wash was repeated twice with extensive homogenization in between each washing step. The cells were finally suspended in a minimal volume (1–2 mg Chl/ml) of the glycerol washing medium and stored on ice. Spinach thylakoids were prepared as previously described (2). Chl was determined according to Arnon (1).

ATPase Complexes. Extraction of the CF_0-CF_1 ATPase complex from *D. bardawil* was performed by a modification of the procedure previously described for the extraction of the higher plant CF_0-CF_1 complex (23). *D. bardawil* cells were collected as described above and washed four times with 10 mM Na pyrophosphate (pH 7.8) buffer. Cells were collected by centrifugation at 14,000*g* for 10 min at 5°C. The solubilization medium contained 20 mM Na-Tricine (pH 8.0), 0.2 M sucrose, 50 mM DTT, 3 mM $MgCl_2$, 0.5% Na cholate, 30 mM octylglucoside, and *D. bardawil* thylakoid membranes (equivalent to 2 mg Chl/ml). $(NH_4)_2SO_4$ was omitted. In order to remove the residual carotene released during the solubilization, 1.0 ml of the Mg-Tricine-sucrose buffer solution was layered on top of the solubilization mixture immediately prior to the high speed centrifugation

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[3] Abbreviations: CF_0-CF_1, chloroplast ATP synthase complex; CF_1, chloroplast coupling factor 1; PMS, phenazine methosulfate; OG, octyl-β-D-glucopyranoside (octylglucoside); FTS, the freezing-thawing-sonication reconstitution technique; CD, the cholate-dilution reconstitution technique; DCCD, dicyclohexylcarbodiimide.

(200,000*g* for 45 min at 3°C in a Beckman type 65 rotor). Most of the orange-colored carotene was either extracted into the upper buffer phase or appeared as a paste on the walls of the tube and was carefully removed at the end of the centrifugation.

The yellowish supernatant, which contained the ATPase, was fractionated with $(NH_4)_2SO_4$ between 30 and 50%, and the precipitate was either stored frozen (crude CF_0-CF_1) or further purified on a sucrose gradient containing 0.2% Triton X-100 plus 0.1% soybean lipids. Other details regarding the purification and the reconstitution of the CF_0-CF_1 complex into proteoliposomes were as previously described (23).

Coupling Factor 1 ATPases. Log-phase cultures of *D. bardawil* (6–10 L) were harvested by centrifugation (600*g*, 5 min) and washed twice in 500 ml buffer containing 40 mM Na-Tricine (pH 7.4), 1.5 M NaCl, and 5 mM $MgCl_2$. After the second wash, the cells were osmotically shocked by resuspension in 250 ml of a hypotonic buffer containing 50 mM Na-Hepes (pH 7.6), 10 mM NaCl, and 1 mM EDTA, and incubated at 4°C for 5.0 min. Membrane fragments were collected by centrifugation (3000*g*, 10 min) and washed four times (250 ml/wash) with 10 mM Na pyrophosphate (pH 7.8) buffer.

CF_1 was extracted by chloroform and purified on a DEAE-Sephadex column essentially according to Selman-Reimer *et al.* (27) with the exception that the dialysis prior to the DEAE-Sephadex purification was omitted.

Spinach (20) and *Chlamydomonas reinhardi* (27) coupling factors were prepared as previously described.

Prior to use, aliquots of the $(NH_4)_2SO_4$ precipitated proteins were centrifuged (12,000*g*, 2.0 min), and the proteins were redissolved in buffer containing 40 mM Na-Tricine (pH 8.0) and 1.0 mM EDTA.

Analytical Methods. Cyclic photophosphorylation was measured by the formation of [γ-^{32}P]ATP from $^{32}P_i$ and ADP after illumination of thylakoid membranes (equivalent to 20 μg Chl/ml) for 1.0 min at either 30°C (*D. bardawil*) or at 23°C (spinach) in a water bath (white light, 100,000 lux). The media contained 20 mM Na-Tricine (pH 8.0), 3.0 mM ADP, 5.0 mM $MgCl_2$, 4.0 mM P_i (containing 0.1–0.2 μCi ^{32}P/μmol), and either 100 μM PMS plus 0.2 to 0.5 mM DTT (*D. bardawil* membranes) or 30 μM PMS (spinach thylakoid membranes) with or without 20% glycerol. The formation of [γ-^{32}P]ATP was determined according to Avron (2).

ATP-P_i exchange was measured by the incorporation of $^{32}P_i$ into ATP. Proteoliposomes containing the CF_0-CF_1 complexes (40–80 μg protein/ml) were incubated for 30 min at 37°C in a medium containing 20 mM Na-Tricine (pH 8.0), 5.0 mM $MgCl_2$, 5.0 mM ATP, 1.0 mM K-phosphate (containing 0.5–1 μCi $^{32}P_i$/μmol), and 2.0 mM DTT with or without 20% glycerol.

Ca-ATPase activation of CF_1 was performed by incubating the enzyme (1–2 mg protein/ml) for 3 min at 62°C in a medium containing 40 mM Na-Tricine (pH 8.0), 1.0 mM EDTA, 5.0 mM ATP, and 5.0 mM DTT, followed by cooling to 23°. The Mg-ATPase activity was assayed in the presence of 25% ethanol (*D. bardawil*, or *C. reinhardi* CF_1) or 30 mM octylglucoside (spinach CF_1).

Unless otherwise stated, standard ATPase reaction mixtures contained in 0.1 ml total volume 0.5 μg protein, 40 mM Na-Tricine (pH 8.0), 1.0 mM EDTA, 6.0 mM $MgCl_2$ or $CaCl_2$, and 5.0 mM [γ-^{32}P]ATP (approximately 0.2 μCi ^{32}P/μmol). Reactions were initiated by the addition of protein, incubated for 2 to 3 min at 37°C, and terminated by the addition of 2 ml of a solution containing 0.8 N $HClO_4$ and 1% (w/v) ammonium molybdate. Extraction of [^{32}P]phosphate was performed as previously described (20).

Other Methods. Ferricyanide reduction was measured in an Aminco-Chance DW2 spectrophotometer by following the light-induced absorption changes at 420 minus 470 nm. 9-Aminoacridine fluorescence quenching (for measurements of the transmembrane ΔpH) was measured in an Eppendorf photometer adapted for fluorescence measurements as previously described (26). Protein was estimated either according to Lowry *et al.* (18) for the CF_0-CF_1-ATPase or according to Bradford (11) for CF_1.

The separation and staining of protein polypeptides were performed by slab SDS-polyacrylamide gel electrophoresis (12.5%) essentially according to Laemmli (17). The polymerization solution contained 0.5% polyacrylamide (mol wt above 5×10^6).

Materials. Detergents and chemicals were obtained from Sigma Chemical Co. [γ-^{32}P]ATP was synthesized by the illumination of chloroplasts in the presence of $^{32}P_i$ and ADP followed by the separation of [γ-^{32}P]ATP on polyethyleneiminocellulose columns according to Magnusson *et al.* (19).

RESULTS

Characterization of ATP Formation Catalyzed by *D. bardawil* Thylakoid Membranes. Ben Amotz and Avron (6) have previously reported that thylakoid membranes isolated from osmotically shocked *D. parva* cells have the capacity to catalyze ATP formation in the light in the presence of different electron transfer mediators; however, the rates that they measured were considerably lower than those obtained from higher plant thylakoid membranes. In order to increase the phosphorylation activity of *Dunaliella* thylakoids, the effect of glycerol was tested.

The presence of glycerol during the phosphorylation assay stimulates the rate of PMS-dependent ATP formation with *D. bardawil* thylakoid membranes but inhibits the activity with spinach thylakoid membranes (Fig. 1). An optimal stimulation of about 2-fold is obtained by 20% (2.75 M) glycerol.

In order to determine if the glycerol stimulation of ATP formation was due to an increase in the electron transfer rate, to a lowered permeability of the thylakoid membrane to protons, or to a direct effect of glycerol on the ATP synthase, the effect of glycerol on the light-driven, PMS-dependent, transmembrane ΔpH formation catalyzed by *D. bardawil* thylakoid membranes (measured by 9-aminoacridine fluorescence quenching [26]) was

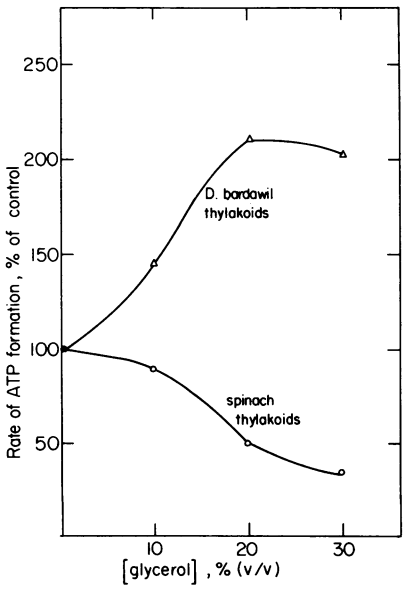

FIG. 1. The differential effect of glycerol on PMS-dependent cyclic photophosphorylation catalyzed by *D. bardawil* and spinach thylakoid membranes. PMS-dependent photophosphorylation was measured in the presence of the indicated glycerol concentrations. The control (100%) rates of ATP formation were 220 and 1700 μmol ATP formed/mg Chl·h with *D. bardawil* and spinach thylakoid membranes, respectively.

determined. However, it was found that glycerol does not affect the magnitude of the light-induced ΔpH formed across the *D. bardawil* thylakoid membranes (not shown). In addition, glycerol slightly inhibits the rates of ferricyanide reduction both in the absence and in the presence of an uncoupler but does not significantly affect the ratio between the uncoupled to coupled rates. For example, without uncoupler, the rates of ferricyanide reduction in the absence and presence of 20% glycerol were 121 and 104 μeq/mg Chl·h, respectively. In the presence of 1 μM nigericin, the rates increased to 288 and 242 μeq/mg Chl·h, respectively. These results suggest, therefore, that the glycerol stimulation of the rate of ATP formation catalyzed by *D. bardawil* thylakoid membranes is not due to a lowering of the membrane permeability to protons or to a stimulation of the electron transfer rate but rather is due to an effect of glycerol on the CF_0-CF_1 ATP synthase.

In comparison to spinach thylakoids, ATP formation catalyzed by *D. bardawil* thylakoid membranes is very sensitive to a high concentration of NaCl (Fig. 2A). NaCl at 300 mM inhibits ATP formation catalyzed by *D. bardawil* thylakoid membranes by over 90% compared to only a 30% inhibition of the rate catalyzed by spinach thylakoid membranes. NaCl inhibition of photophosphorylation catalyzed by *D. bardawil* thylakoid membranes is not dependent on the presence or absence of glycerol (not shown). However, 300 mM NaCl does not affect the light-driven, PMS-dependent, ΔpH formation catalyzed by *D. bardawil* thylakoid membranes, indicating that salt does not alter the efficiency of the rate of electron transfer or the proton permeability of the membrane. Photophosphorylation catalyzed by *D. bardawil* thylakoid membranes was, therefore, routinely measured in a medium containing 20% glycerol and low salt. The opposite effects of glycerol and of NaCl on photophosphorylation also strongly suggest that the glycerol-induced stimulation is not due to an osmotic effect.

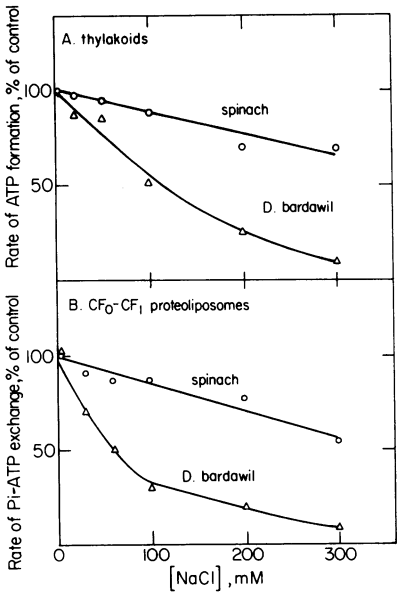

FIG. 2. The differential sensitivity to NaCl of photophosphorylation and of the rates of ATP-P_i exchange catalyzed by preparation from *D. bardawil* and spinach. Cyclic photophosphorylation (A) was measured in the presence (*D. bardawil*) or absence (spinach) of 20% glycerol at the indicated NaCl concentrations as described under "Materials and Methods." The control (100%) rates of ATP formation were 533 and 455 μmol ATP/mg Chl·h with *D. bardawil* and spinach thylakoid membranes, respectively. ATP-P_i exchange activity of CF_0-CF_1 containing proteoliposomes prepared by the FTS reconstitution technique was measured as described in the text. The control rates of ATP-P_i exchange were 140 and 20 nmol ATP formed/mg protein·min for spinach and *D. bardawil* complex containing liposomes, respectively.

Inhibitors of the higher plant CF_0-CF_1 ATP synthase also inhibit PMS-dependent photophosphorylation catalyzed by *D. bardawil* thylakoid membranes (not shown). It is of interest to note that tentoxin, a species-specific inhibitor of photophosphorylation catalyzed by higher plant thylakoid membranes, also inhibits photophosphorylation catalyzed by *D. bardawil* thylakoid membranes, albeit at relatively high concentrations. The K_{50} value for inhibition is 3 μM (compared to 0.2 μM with spinach thylakoid membranes assayed under similar conditions), whereas complete inhibition requires about 100 μM tentoxin. In addition, antisera directed against the spinach CF_1 strongly inhibit photophosphorylation catalyzed by *D. bardawil* thylakoid membranes, suggesting an intimate structural similarity between the two enzymes (not shown).

Characterization of the *D. bardawil* CF_1 ATPase. In contrast to the *C. reinhardi* ATPase, but similar to the spinach enzyme, the ATPase activity of the *D. bardawil* CF_1 is completely latent. Although the *D. bardawil* ATPase activity is not induced by proteolytic digestion of the enzyme, the latency is released by selective organic solvents or preincubation of the enzyme at 62°C for 3.0 min in the presence of 5.0 mM DTT and 10 mM ATP.

Organic solvents induce a Mg^{2+}-dependent ATPase activity. Among the most effective solvents tested were ethanol and methanol. Optimal stimulation of the rates of the ATPase activity was obtained at ethanol and methanol concentrations equivalent to 25 and 35% (v/v), respectively (Fig. 3A). These results are similar to those previously found for the *C. reinhardi* (27) and spinach CF_1 (25). In the presence of 25% ethanol, the K_m for MgATP is 1.2 mM and V_{max} is approximately 60 to 80 units/mg protein. The detergent OG, which stimulates a Mg^{2+}-dependent ATPase activity with the higher plant CF_1 (22; Fig. 3B), also stimulates a Mg^{2+}-dependent ATPase activity with the *D. bardawil* CF_1 (at similar concentrations, 30–40 mM). However, a comparison of Figure 3, A and B, demonstrates that ethanol is more effective in stimulating the *D. bardawil* ATPase while OG is more effective in stimulating the Mg-ATPase of the higher

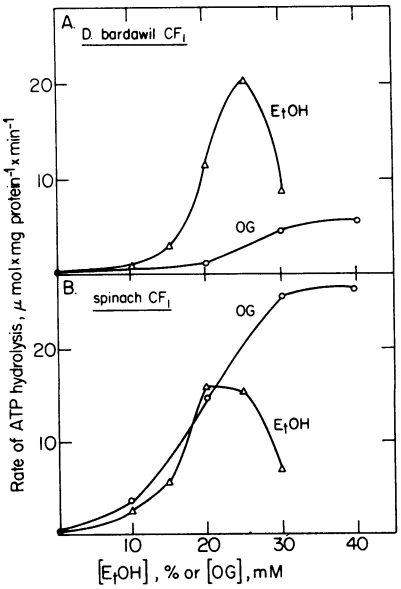

FIG. 3. Ethanol and octylglucoside activation of the Mg^{2+}-dependent ATPase activity of CF_1 isolated from *D. bardawil* and spinach. The Mg^{2+}-dependent ATPase activity of the chloroform-extracted CF_1 from *D. bardawil* (A) and spinach (B) was assayed in the presence or absence of the indicated concentrations of OG or ethanol as described in "Materials and Methods."

plant CF_1.

Heat treatment of the *D. bardawil* CF_1 induces a Ca^{2+}-dependent ATPase activity, in a manner similar to that of other higher (vascular) plant coupling factors (12). Whereas the solvent-induced, Mg^{2+}-dependent ATPase activity is reversible upon the removal of the solvent, the heat-induced, Ca^{2+}-dependent activation of the *D. bardawil* ATPase activity is irreversible. The latter absolutely requires the presence of both DTT and ATP during the activation process. The addition of DTT after the heat treatment has no effect on the rate of the ATPase.

A comparison of the glycerol sensitivity of the solvent-induced algal and OG-induced spinach Mg-ATPase activities is shown in Figure 4A. Figure 4B shows a comparison of the glycerol sensitivity of the heat-activated, Ca-ATPase activity of the *D. bardawil* and spinach CF_1. The ethanol-induced, Mg-ATPase activity of the *D. bardawil* CF_1 is the least sensitive to glycerol (in fact, a small, 10 to 15%, but reproducible, stimulation is usually observed at about 5% [v/v] glycerol). The Mg-ATPase activities of both the spinach (OG-induced) and the *C. reinhardi* (ethanol-induced) coupling factors are more sensitive than the *D. bardawil* CF_1 to glycerol inhibition, the *C. reinhardi* enzyme being the most sensitive. On the other hand, the heat-activated Ca-ATPase activity of both the *D. bardawil* and spinach coupling factors are very sensitive to glycerol inhibition. Under the conditions used, 50% inhibition was obtained at about 5 to 8% (v/v) glycerol.

Isolation of the CF_0-CF_1 ATP Synthase from *D. bardawil*. Isolation of the CF_0-CF_1 ATP synthase complex from *D. bardawil* thylakoid membranes was achieved by a modification of the procedure previously used for the isolation of the higher plant enzyme (23). The high carotene content (up to 30% of the total dry weight of the alga [13]) interferes with the purification of the enzyme. For this reason, *D. bardawil* was cultured on a rich growth medium which inhibits the biosynthesis of carotene (8). The solubilization procedure was also modified in order to remove the carotene globules released during the detergent solubilization. This included layering buffer on top of the solubilization mixture to extract the residual carotene as described under "Materials and Methods." Another modification introduced into the isolation of the *D. bardawil* CF_0-CF_1 complex was to omit the $(NH_4)_2SO_4$ during solubilization. (The presence of $(NH_4)_2SO_4$ is essential for the isolation of the higher plant enzyme. Attempts to solubilize the spinach CF_0-CF_1 complex in the absence of salt resulted in a massive solubilization of Chl and Chl-binding proteins, both of which interfere with the purification of the complex [U. Pick, unpublished results].) Table I shows that, if the *D. bardawil* complex is solubilized in the presence of 10% $(NH_4)_2SO_4$ ATP-P_i exchange activity catalyzed by CF_0-CF_1 containing proteoliposomes is inhibited over 80%. The inhibition by $(NH_4)_2SO_4$ is not due to an interference in the solubilization of the complex *per se* since the same recovery of solvent-activated-ATPase activity is obtained in the extract regardless of $(NH_4)_2SO_4$ (data not shown). It is possible, however, that the presence of salt during the solubilization causes a specific inactivation of the CF_0 or a disruption in the coupling between CF_0 and CF_1.

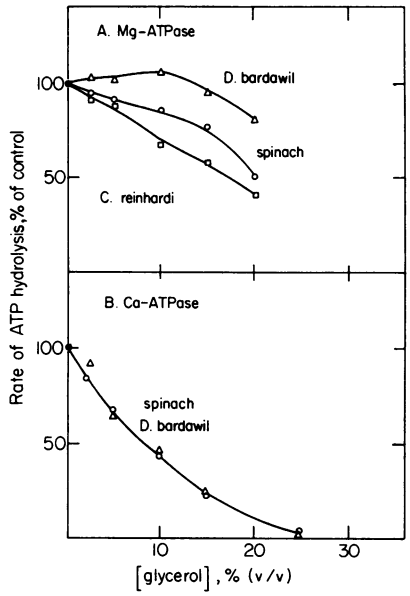

FIG. 4. The differential effects of glycerol on the Mg^{2+}- and Ca^{2+}-dependent ATPase activities of CF_1 preparations isolated from *D. bardawil*, *C. reinhardi*, and spinach. Mg^{2+}-dependent ATPase activity (A) was measured in the presence of 25% (v/v) ethanol (*D. bardawil* and *C. reinhardi* CF_1) or 30 mM octylglucoside (spinach). The Ca^{2+}-dependent ATPase (B) of heat-activated CF_1 from *D. bardawil* or spinach was measured in the presence of the indicated glycerol concentrations as described under "Materials and Methods."

The purified enzyme may be reconstituted into proteoliposomes with soybean phospholipids by either the freezing-thawing-sonication technique (16) or the cholate-dilution technique (24). The FTS-reconstituted CF_0-CF_1 proteoliposomes catalyze ATP-P_i exchange rates of about 50 nmol/mg protein·min and the activity is inhibited by protonophoric uncouplers, DCCD and tentoxin. The apparent K_m values for ADP (0.8 mM) and P_i (0.75 mM) are similar to the K_m values for the spinach enzyme. Reconstitution of the purified *D. bardawil* CF_0-CF_1 complex with bacteriorhodopsin and soybean phospholipids (1:2:80 weight ratio) by the FTS procedure yields proteoliposomes which catalyze ATP synthesis in the light. The rates are linear for at least 60 min and ranged from 10 to 20 nmol ATP/mg CF_0-CF_1 protein·min at 30°C.

Polypeptide Subunit Composition of the Purified *D. bardawil* CF_1 and CF_0-CF_1 complexes. A comparison of the polypeptide subunit composition of the CF_0-CF_1 complex and of CF_1 from spinach (lanes 1 and 2) and from *D. bardawil* (lanes 3 and 4) is shown in Figure 5. Four distinct bands can be seen in the chloroform-released preparations (lanes 1 and 4) while seven major bands (as well as several minor contaminants) are present in both CF_0-CF_1 complexes (lanes 2 and 3). However, the relative migration of several subunits of the *D. bardawil* complex differs substantially from that of spinach. The most striking difference is the location of the α subunit which migrates ahead of the β subunit in *D. bardawil*. The identification of the α and β subunits of the *D. bardawil* CF_1 in comparison to spinach CF_1 was made by immune cross-reactivity experiments using monospecific antisera and by chemical modification (28). Other differences between the two enzymes are the slower migration of the *D. bardawil* δ subunit and the faster migration of the *D. bardawil* ϵ subunit with respect to the two larger polypeptide subunits of

Table I. *Inhibitory Effect of Ammonium Sulfate When Present during the Solubilization on the Activity of the Reconstituted D. bardawil CF_0-CF_1 ATPase Complex*

The *D. bardawil* CF_0-CF_1 ATPase was solubilized in the presence of the indicated $(NH_4)_2SO_4$ concentrations, and samples from the crude enzyme fractions (30–50% saturation of $(NH_4)_2SO_4$) were reconstituted into proteoliposomes by the FTS procedure and assayed for ATP-P_i exchange.

$[(NH_4)_2SO_4]$	Rate of ATP-P_i Exchange
% (w/v)	*nmol/mg protein·min*
0	50
5	15
10	7
20	4

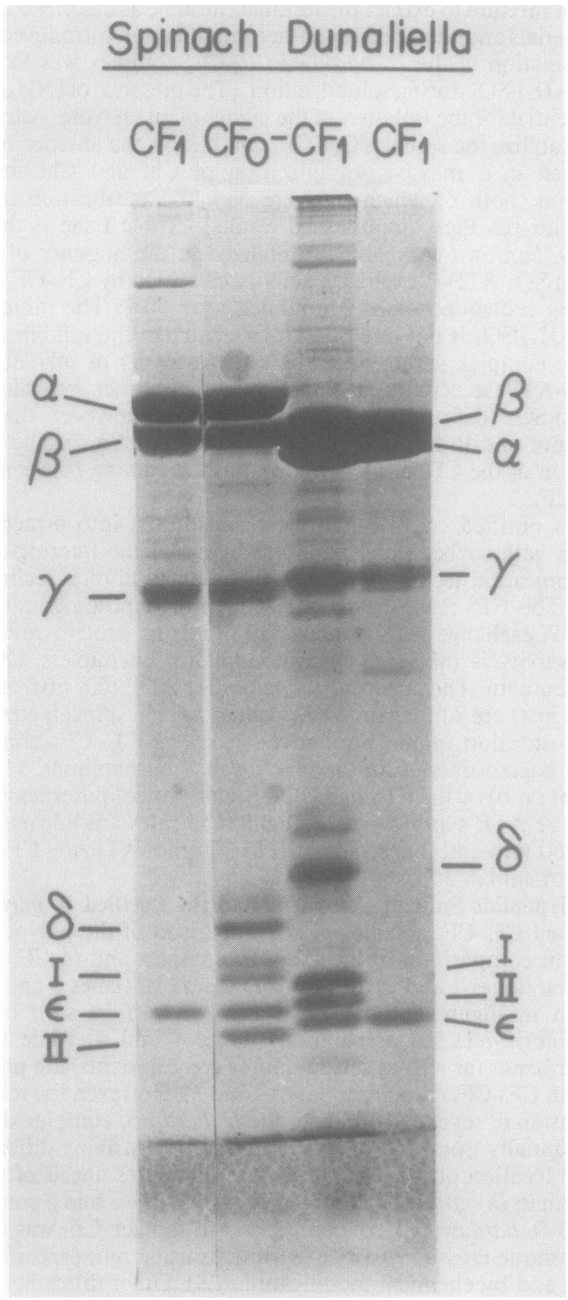

FIG. 5. Comparison of the CF_1 and CF_0-CF_1 polypeptides from *D. bardawil* and spinach by SDS-polyacrylamide gel electrophoresis. The chloroform-extracted CF_1 from spinach (lane 1) and *D. bardawil* (lane 4) and the purified CF_0-CF_1 ATPases from spinach (lane 2) and *D. bardawil* (lane 3) were isolated as described under "Materials and Methods" and separated on 12.5% SDS-polyacrylamide gel (17). Samples containing 30 μg protein were applied to each slot. Greek letters designate CF_1 subunits and Roman letters designate CF_0 subunits.

the *D. bardawil* CF_0 (I and II). The DCCD-binding protein (subunit III) is not seen in this gel due to its poor staining. The apparent mol wt of the *D. bardawil* CF_0-CF_1 subunits β, α, γ, δ, I, II, and ε (calculated by comparison with the migration of a set of polypeptide markers) are 52, 49, 35, 21, 17.8, 17, and 16.5 kD, respectively.

The Effects of Glycerol and Salt on the Activity of the *D. bardawil* CF_0-CF_1 Complex. Figure 6 shows that glycerol stimulates the rate of ATP-P_i exchange in proteoliposomes containing the *D. bardawil* CF_0-CF_1 complex, but the magnitude of stimulation depends on the technique used for reconstitution. Glycerol at 10 to 20% only slightly stimulates (about 10%) the rate of ATP-P_i exchange with proteoliposomes prepared by the FTS technique in which the lipid to protein ratio is high (50–100). However, glycerol at 20% greatly enhances the rate of ATP-P_i exchange with CF_0-CF_1 proteoliposomes prepared by the CD technique. These proteoliposomes have a lower lipid to protein ratio (8–10) and, in the absence of glycerol, catalyze a lower rate of ATP-P_i exchange (between 10 and 50% of the maximal rate). At high glycerol concentrations, the activity of *D. bardawil* CF_0-CF_1 complex containing proteoliposomes is only slightly inhibited irrespective of the reconstitution procedure whereas the activity of proteoliposomes containing the spinach enzyme is strongly inhibited by glycerol (K_{50} = 15%).

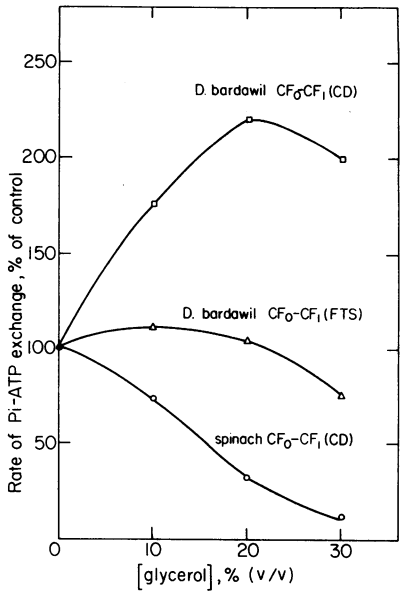

FIG. 6. Glycerol titration curve for the ATP-P_i exchange activity catalyzed by *D. bardawil* and spinach CF_0-CF_1 containing proteoliposomes. CF_0-CF_1 proteoliposomes were reconstituted by CD or by FTS reconstitution techniques, and ATP-P_i exchange was measured in the presence of the indicated glycerol concentrations as described in "Materials and Methods." The control (100%) rates of ATP-P_i exchange were 170, 20, and 45 nmol ATP/mg protein·min with spinach, *D. bardawil* (CD) and *D. bardawil* (FTS) complex containing proteoliposomes, respectively.

In order to determine if the low glycerol stimulation and the high glycerol inhibition of the rate of ATP-P_i exchange catalyzed by *D. bardawil* CF_0-CF_1 containing proteoliposomes are reversible, the reconstituted enzyme was preincubated with or without glycerol and subsequently assayed under different conditions. These results, summarized in Table II, demonstrate that preincubation of CD-proteoliposomes with glycerol does not stimulate the rate of ATP-P_i exchange. This stimulation requires the presence of glycerol during the assay. Similarly, preincubation of FTS-proteoliposomes with 50% glycerol does not inhibit the rate of ATP-P_i exchange to the same extent as does 50% glycerol in the assay. These results indicate that both the low glycerol stimulation and the high glycerol inhibition of the rate of ATP-P_i exchange catalyzed by *D. bardawil* CF_0-CF_1 containing proteoliposomes are reversible effects.

The stimulation of ATP-P_i exchange in proteoliposomes containing the *D. bardawil* CF_0-CF_1 complex prepared by the CD method is not unique to glycerol and can also be obtained by comparable concentrations of other polyols such as ethylene glycol, sorbitol, or sucrose, as shown in Table III.

The activity of *D. bardawil* CF_0-CF_1 containing proteolipo-

Table II. *The Reversibility of the Effects of Glycerol on the Rate of ATP-P_i Exchange Catalyzed by Proteoliposomes Containing the D. bardawil CF_0-CF_1 Complex*

D. bardawil CF_0-CF_1 containing proteoliposomes were prepared either by CD or by FTS techniques. The vesicles were incubated for 5 min at 23°C in 1 ml containing 40 mM Na-Tricine (pH 8.0), 0.1 mM EDTA, and 0, 20, or 50% glycerol. The incubation was terminated by passing the proteoliposomes through Sephadex columns by the centrifugation procedure (21). The columns were pre-equilibrated with the same buffer at the indicated glycerol concentrations. Control rates (100%) of ATP-P_i exchange were 52.4 and 47 nmol ATP formed/mg protein·min in FTS and CD proteoliposomes, respectively.

Type of Reconstitution	Glycerol Concentration			ATP-P_i Exchange,
	Preincubation	Sephadex	Assay	
	% (v/v)			% control
CD	0		20	100
	20	20	20	86
	20	0	0	7.3
	0	0	0	2.5
FTS	20		20	100
	20	20	20	75
	50	20	20	66
	50	50	50	25

Table III. *Polyol Specificity for the Stimulation of the Rate of ATP-P_i Exchange Catalyzed by D. bardawil CF_0-CF_1 Containing Proteoliposomes*

D. bardawil CF_0-CF_1 containing proteoliposomes were prepared by the CD reconstitution technique, and ATP-P_i exchange was measured in the absence or presence of 10 or 20% ethylene glycol (v/v), glycerol (v/v), sorbitol (w/v), or sucrose (w/v). The control (100%) rate of ATP-P_i exchange was 9 nmol ATP formed/mg protein·min.

	ATP-P_i Exchange with Polyol at		
	0	10%	20%
	% control		
Ethylene glycol	100	138	138
Glycerol	100	145	167
Sorbitol	100	136	166
Sucrose	100	115	150

somes is also more sensitive to high salt than is the activity of proteoliposomes containing the spinach enzyme, as demonstrated in Figure 2B. NaCl inhibited the rate of ATP-P_i exchange by 50% at 60 mM NaCl with *D. bardawil* CF_0-CF_1 complex containing proteoliposomes whereas 300 mM NaCl was required for 50% inhibition with spinach CF_0-CF_1 complex containing proteoliposomes.

The effects of salt and of glycerol on the purified *D. bardawil* and spinach enzymes are, therefore, generally similar to the effects of salt and glycerol on photophosphorylation and may indicate that the differences in the sensitivity of *D. bardawil* and spinach thylakoid membranes to salt and glycerol are at least in part due to the different properties of the CF_0-CF_1 ATP synthases.

DISCUSSION

The results presented here demonstrate that *D. bardawil* thylakoid membranes, CF_0-CF_1 complex, and CF_1 exhibit a high tolerance to high glycerol concentrations and even, in some cases, a glycerol dependence when compared to similar systems found in other higher plants and algae. These findings suggest that the photosynthetic apparatus in *D. bardawil* is well adapted to function at the high glycerol concentrations which are accumulated internally in the cells under physiological growth conditions (7, 10). Very few comparisons of the glycerol sensitivity of various *Dunaliella* enzymes with other high plant enzymes have been performed in the past. Ben Amotz (4) found, for example, that whereas the rate of electron transfer from water to Diuat catalyzed by lettuce thylakoid membranes is slightly inhibited by 3 M glycerol, the rate of electron transfer catalyzed by *D. parva* thylakoid membranes was stimulated. The observation that 20% glycerol (about 2.7 M) stimulates ATP formation by *D. bardawil* thylakoid membranes is consistent with this observation. The glycerol concentration (2.7 M) that optimally stimulates photophosphorylation with *D. bardawil* thylakoid membranes is close to the optimal external osmolarity required for maximal photosynthetic activity of whole *Dunaliella* cells (7). Tolerance to high glycerol concentrations is found not only for reactions catalyzed by *D. bardawil* thylakoid membranes but also for the reactions catalyzed by the purified *D. bardawil* CF_0-CF_1 complex when reconstituted into proteoliposomes (Fig. 6) and for the Mg^{2+}-dependent ATPase activity of the *D. bardawil* CF_1 (Fig. 4A). This suggests that the structure of the *D. bardawil* CF_0-CF_1 ATP synthase has been modified in comparison to the spinach or the related alga *C. reinhardi* complexes. This is indeed indicated by the altered migration of the *D. bardawil* CF_0-CF_1 subunits on SDS-polyacrylamide gels reported here, by an altered amino acid composition, and by an increased sensitivity to various inhibitors (to be reported elsewhere).

There are some intriguing observations regarding the effects of glycerol on the *D. bardawil* ATPase. These include the differential effect of glycerol on the rate of ATP-P_i exchange depending upon the method chosen to reconstitute proteoliposomes (Fig. 6), and the differential sensitivity of the induced *D. bardawil* CF_1 ATPase activity depending upon the method chosen for induction (Fig. 4).

The differential effect of glycerol on the stimulation of the rate of ATP-P_i exchange in CD and FTS proteoliposomes catalyzed by the *D. bardawil* CF_0-CF_1 complex may be related to the different lipid/protein ratios used in the two reconstitution procedures. The highest rates of ATP-P_i exchange catalyzed by the spinach CF_0-CF_1 complex are obtained by the CD reconstitution technique (U. Pick, unpublished observations). This procedure, however, is not very effective in the preparation of active proteoliposomes containing the *D. bardawil* CF_0-CF_1 complex. The FTS procedure produces proteoliposomes with a much higher activity. The ATP-P_i exchange activity of the *D. bardawil* enzyme apparently requires a higher lipid to protein ratio in the absence of glycerol, but the activity may be improved by glycerol at a limiting lipid to protein ratio. This interpretation implies that the effect of glycerol is at least partly at the level of lipid-protein interactions.

A high tolerance of the *D. bardawil* CF_1 to glycerol is also apparent with the soluble CF_1. The Mg^{2+}-dependent ATPase activity induced by solvents is virtually unaffected by glycerol concentrations as high as 20 to 30% (v/v). However, the differences between the glycerol sensitivity of the *D. bardawil* and spinach enzymes are smaller than those observed for reactions catalyzed by thylakoid membranes and the CF_0-CF_1 containing proteoliposomes. In contrast, the heat-induced Ca^{2+}-dependent ATPase activity of both enzymes is equally sensitive to glycerol inhibition (Fig. 4B). It may be noteworthy that the OG-activated, Ca-ATPase activity of spinach CF_1 is also more sensitive to high ionic strength in comparison to the OG-dependent, Mg-ATPase ctivity (22). A possible explanation for these differences is that soluble CF_1 (in the absence of organic solvents or OG) exists in a distorted conformation which alters its structural and catalytic properties (allotopy). We have previously suggested that OG and organic solvents may induce a structural change in solbule CF_1

converting it to a form that resembles its native conformation and then restores its catalytic properties (22). The differential sensitivity of the *D. bardawil* CF_1 to glycerol in the presence or absence of alcohols would be consistent with this view.

Although a careful comparison between *Dunaliella* and other higher plant enzymes with respect to their sensitivities to salt has not been performed, it has been demonstrated that the activities of *Dunaliella* membranes, as well as some soluble enzymes are sensitive to NaCl (6, 7, 10). The results shown in Figure 2 are consistent with previous results and demonstrate that *Dunaliella* chloroplasts are even more sensitive to NaCl than other higher plant chloroplasts. This might suggest that the *Dunaliella* photosynthetic system is adapted to a low ionic strength environment.

Direct measurements of the internal NaCl content of *Dunaliella* cells and of the permeability of the *Dunaliella* outer membrane to NaCl are controversial. Indirect, earlier evidence (15) and more recent observations (14) suggest that the outer membrane of *Dunaliella* is quite permeable to NaCl and that the internal salt concentration in the alga is high. In contrast more recent direct measurements of the salt content of *Dunaliella* cells grown in high salt suggest that the internal NaCl concentration in *Dunaliella* is low (M. Avron, personal communication).

The results reported here demonstrate that the photosynthetic system of *D. bardawil* is adapted to a high glycerol, low salt medium and therefore is consistent with the idea that under normal growth conditions (high NaCl), *Dunaliella* cells contain high glycerol and low salt internally. The structural changes, reflected in the altered migration of *D. bardawil* CF_0-CF_1 subunits on SDS-PAGE may be related to this adaptation.

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LITERATURE CITED

1. ARNON DI 1949 Copper enzymes in isolated chloroplasts: polyphenol oxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
2. AVRON M 1960 Photophosphorylation by Swiss chard chloroplasts. Biochim Biophys Acta 40: 257–272
3. AVRON M 1961 Photophosphorylation as a tool for the synthesis of specifically labelled nucleotides. Anal Biochem 2: 535–543
4. BEN-AMOTZ A 1973. Photosynthetic and osmoregulation mechanisms in the halophilic alga *Dunaliella parva*. Ph.D. thesis. Weizmann Institute of Science, Rehovot, Israel
5. BEN-AMOTZ A 1975 Adaptation of the unicellular alga *Dunaliella parva* to saline environment. J Phycol 11: 50–54
6. BEN-AMOTZ A, M AVRON (1972) Photosynthetic activities of the halophilic alga *Dunaliella parva*. Plant Physiol 49: 240–243
7. BEN-AMOTZ A, M AVRON 1973 The role of glycerol in osmotic regulation of the halophilic alga *Dunaliella parva*. Plant Physiol 51: 875–878
8. BEN-AMOTZ A, A KATZ, M AVRON 1982 Accumulation of β-carotene in halotolerant algae: purification and characterization of β-carotene rich globules from *Dunaliella bardawil* (Chlorophyceae). J Phycol 18: 529–537
9. BINDER A, R BACHOFEN 1979 Isolation and characterization of a coupling factor 1 ATPase of the thermophilic blue-green alga (Cyanobacterium) *Mastigocladus laminosus*. FEBS Lett 104: 66–70
10. BOROWITZKA LF, AD BROWN 1974 The salt relations of marine and halophilic species of the unicellular green alga *Dunaliella*. Arch Microbiol 96: 37–52
11. BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
12. FARRON F, E RACKER 1970 Studies on the mechanism of the conversion of coupling factor 1 from chloroplasts to an active adenosine triphosphatase. Biochemistry 9: 3829–3836
13. FRIED A, A TIETZ, A BEN-AMOTZ, W EICHENBERGER 1982 Lipid composition of the halotolerant alga *Dunaliella bardawil*. Biochim Biophys Acta 713: 419–426
14. GIMMLER H, R SCHIRLING 1978 Cation permeability of the plasmalemma of the halotolerant alga *Dunaliella parva*. II. Cation content and glycerol concentration of the cells as dependent upon external NaCl concentration. Pflanzenphysiol 87: 435–444
15. GINZBURG M 1969 The unusual membrane permeability of two halophilic unicellular organisms. Biochim Biophys Acta 173: 370–376
16. KASAHARA M, PC HINKEL 1977 Reconstitution and purification of the D-glucose transporter from human erythrocytes. J Biol Chem 252: 7384–7390
17. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of the bacteriophage T_4. Nature 227: 680–685
18. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
19. MAGGNUSON RP, RE MCCARTY 1976 Light-induced exchange of nucleotides into coupling factor 1 in spinach chloroplast thylakoids. J Biol Chem 251: 7417–7422
20. NELSON N 1980 Coupling factors from higher plants. Methods Enzymol 69: 301–313
21. PENEFSKY HS 1977 Reversible binding of P_i by beef heart mitochondrial adenosine triphosphatase. J Biol Chem 252: 2891–2899
22. PICK, U, S BASSILIAN 1982 Activation of magnesium ion specific adenosine triphosphatase in chloroplast coupling factor 1 by octylglucoside. Biochemistry 24: 6144–6152
23. PICK U, E RACKER 1979 Purification and reconstitution of the *N,N'*-dicyclohexylcarbodiimide-sensitive ATPase complex from spinach chloroplasts. J Biol Chem 254: 2793–2799
24. RACKER E, TF CHIEN, A KANDRACH 1975 A cholate-dilution procedure for the reconstitution of the Ca pump, Pi-ATP exchange, and oxidative phosphorylation. FEBS Lett 55: 14–18
25. SAKURAI H, K SHINOHARA, T HISABORI, K SHINOHARA 1981 Enhancement of adenosine triphosphatase activity of purified chloroplast coupling factor 1 in an aqueous organic solvent. J Biochem 90: 95–102
26. SCHULDINER S, H ROTTENBERG, M AVRON 1972 Determination of delta-pH in chloroplasts. II. Fluorescent amines as a probe for the determination of delta-pH in chloroplasts. Eur J Biochem 25: 64–70
27. SELMAN-REIMER S, S MERCHANT, BR SELMAN 1981 Isolation, purification, and characterization of coupling factor 1 from *Chlamydomonas reinhardi*. Biochemistry 20: 5476–5482
28. SELMAN-REIMER S, M FINEL, U PICK, BR SELMAN 1983 A partial characterization of the halotolerant green algal *Dunaliella bardawil* CF_1 ATPase and its modification by fluorescein isothiocyanate. *In* C. Sybesma, ed, Proceedings of the 6th International Congress on Photosynthesis, Brussels. Belgium. In press